Detection of prosthetic-hip infections using real-time PCR

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Abstract

Diagnosis of infection after total hip replacement is known to be difficult. Isolation of the bacterial pathogen can give false negative results with current culture methods. Molecular-based methods, as Polymerase Chain Reaction (PCR), may help detect bacteria where culture fails; however, contamination with bacterial DNA when using universal bacterial primers has for a long time been a recognized problem. In this study we describe our attempts of designing a new, rapid PCR method for detection of bacteria in synovial fluid.
During the development of this method both the QIAamp® DNA Mini Kit and the MagNA Pure LC Microbiology Kit M^{GRADE} was tested for its extraction reliability.
Hundred and fifteen synovial fluid samples were analysed for *Staphylococcus aureus* and methicillin-resistant staphylococci DNA, using the *nuc* and *mecA* gene respectively. The synovial fluids were also tested with universal bacterial primers and probe. The PCR results were then compared to the standard culture results.
This study shows that culture for S.aureus and methicillin-resistant staphylococci was concordant with the results for *nuc* and *mecA* PCR. The universal PCR protocol detects more bacteria (58%) than the culturing method, but also presents many inconclusive results difficult to interpret. In our protocol the MagNA Pure LC Microbiology Kit M^{GRADE} showed to be more reliable as DNA-extraction method.
Our goal in this study was to develop a better and more reliable diagnostic method for the complicated hip replacement infections. The protocol shows the high potentials of the molecular-based methods, but the bacterial DNA contamination issues using broad-range PCR still remain a problem, and more research is needed to overcome this obstacle.

Introduction

In hip arthroplasty, diagnosis of infection is still an unsolved problem. Uniform criteria for the diagnosis of infection have not been established [1]. Almost half a million total hip and knee arthroplasties are annually performed worldwide [2]. Most patients restore full function, but a few per cent suffers post-operative complications. The main reasons for arthroplasty failure are aseptic loosening, infection and osteolysis [3]. These complications are often difficult to treat, with long term pain and discomfort for the patients. The small, but significant number of patients suffering from an undetected infection will not receive proper treatment and face chronic hip failure. The calculated cost of each total hip arthroplasty revision varies from US$ 5 600 to 28 700 [4].

Aseptic loosening is a diagnosis of exclusion, and the aetiology is unknown [5] [6]. Clarke et al [7] showed in their study that tissue samples taken from arthroplasties considered to be aseptically loose implants, contained significantly more often bacterial DNA than their controls. Unfortunately they had contamination issues giving the study high rates of false-positives (21%).

The current method to isolate the etiologic agent is culturing of aspirated joint fluid or tissue obtained at revision. Culturing can be insensitive and time-consuming [8]. The method depends on the etiologic agent and the specimens that are submitted for culture. Multiple specimens should be obtained and rapidly cultured in appropriate media. One suspects that biofilm, few bacteria, fastidious, slow growing and/or anaerobic microorganisms, extended transportation, inadequate preservation and antibiotic treatment all take part in causing false negative culturing results [6].
The spectrum of microbial agents capable of causing prosthetic joint infection is unlimited, the most common referred in table 1. The spectrum also includes organisms ordinarily considered ”contaminants” of cultures, such as corynebacteria, propionebacteia, and *Bacillus* spp. Rarely have infections with fungi (particularly *Candida*) or mycobacteria been described [9].

In data collected by the Norwegian arthroplasty register aseptic loosening was recognized as the primary cause of revision [3]. After starting a more aggressive antibiotic treatment during surgery, the aseptic loosening rate also went down, suggesting that low grade infection is the primary cause, and often misclassified as aseptic loosening.

Polymerase Chain Reaction (PCR) is now emerging as a diagnostic tool of great value in bacterial detection [10]. PCR has successfully detected nonculturable bacteria in a variety of infections, such as meningitis [11] and septic arthritis [12]. Molecular-based methods, as PCR, bring hope where culture fails. The resent development in the PCR-technology is the real-time PCR which is time saving and less exposed to carryover contamination.
Researchers worldwide are currently trying to develop new molecular methods to decrease detection time and increase assay sensitivity in arthroplasty infections. The well preserved DNA sequence of the eubacterial 16S rRNA gene has been used to secure detection of all bacteria. PCR can amplify extremely low levels of bacterial DNA to detectable levels, making it in theory, a perfect method for the low-grade hip infections.
Several problems in reaching this goal have, however, been described, in particular problems with synovial fluid as a sample material and the bacterial DNA contamination issues with broad-range PCR.

Bacterial DNA is everywhere. The highly sensitive PCR-technique with its enormous amplification power gives this sensitive method high potential, but also demands full sterility. Every known decontamination method has been tested, including UV-irradiation, filtering, 8-MOP-treatment and incubation with various enzymes [10] [13]. None of these have yet convinced as a bacterial DNA contamination problem solving method.

In this study we describe our experiences with the attempt to establish a PCR method for detection of bacteria in hip replacement infections. We have tested decontamination methods described as successful and different PCR protocols attempting to detect only the truly infected cases.

Material and methods

Patients. Hundred-and six patients who underwent one or more revision hip arthroplasty from 1996-2002 was included in the study. The group of patients consisted of 78 women and 28 men, with a mean age of 72.5 years (range, 31-96 years). Reasons for revision surgery included infection, suspicion of infection, mechanical failure (aseptic loosening) and osteolysis.

Sample collection. Hundred-and fifteen synovial fluid samples from 106 patients was collected per-operative in an ultra sterile, laminar air flow equipped operating theatre. The samples were drawn after exposing the hip joint and before opening the hip capsule and collected on 3 ml standard EDTA-tubes which were retrospectively analyzed for bacterial DNA. Until PCR testing the samples were stored at -70 °C. From all revisions, at least 3 samples were cultured for common bacteria using standard techniques and protocols (se below).

Culturing. All revisions samples were cultured for common bacteria using standard techniques and protocols at the Department of Microbiology, Ulleval University Hospital, Oslo, Norway. Bacterial culture included aerobic and anaerobic protocol. For aerobic culture chocolate agar and enrichment were used and the cultures were incubated in 5% carbon dioxide at 35ºC for 7 days. The anaerobic culture was cultured at 35ºC for 5 days. 3 samples were not anaerobic cultured.

Culture of revision samples where defined as positive if the same bacteria were found in at least 2 different samples. In most cases, more than 5 revision samples were taken for culture, including synovial fluid where this was abundant. 34 synovial fluids were cultured aerobic and anaerobic, 10 only aerobic. Synovial fluids with minimum three revisions samples for culturing were included in the study, as recommended by Zimmerli, Trampuz et al [1].

Bacterial strains. The species and American Type Culture Collection (ATCC) of 19 common pathogenic microorganisms (Table 2) were grown in standard cultures, and genomic DNA was extracted using the QIAamp® DNA Mini Kit (Qiagen, GmbH, Germany) as described below.

DNA extraction. Genomic DNA was extracted from synovial fluid specimens using QIAamp® DNA Mini Kit, following Protocol D with some modification. Bacterial cell pellet from 300 µl fluids was resuspended in a combination of 20 mg/ml lysozyme and 0.2 mg/ml lysostaphin (Sigma-Aldrich Co., St.Louis, MO, USA) solution and incubated for 1 hour at 37^{o}C to ensure complete lysis of all bacterial cells. Finally, the elution step was performed by adding 100 µl molecular biology reagent water (Sigma-Aldrich Co) and incubated for 5 min to increase DNA yield. To check for cross-contamination of samples during DNA extraction and bacterial DNA contamination of the reagents, negative controls were included after every second clinical sample. These negative controls consisted of sterile phosphate-buffered saline (PBS) and were handled in the same way as the patient samples.

Bacterial DNA from some of the samples was also extracted using MagNA Pure LC Microbiology Kit M^{GRADE} (Roche Applied Science, Penzberg, Germany) according to the manufacturer´s instructions, with some modifications. To ensure the highest possible sensitivity, bacterial cells were first pelleted from 100 - 600 µl synovial fluid, most of the supernatant was then discarded and the concentrated material and lysis buffer mix including

proteinase K was incubated for 30 min instead of 10 min. The M^{GRADE} reagents are designed to perform isolation of highly purified bacterial genomic DNA from a variety of sample materials. They are tested for bacterial and fungal DNA contamination and no such contamination was detected. One negative DNA extraction control for every seventh clinical sample was included in every run.

To validate the DNA extraction procedure, a positive control prepared from one S.*aureus* culture positive synovial fluid sample was treated exactly as patient sample in each run. Special precautions were taken to avoid bacterial DNA contamination and carry over contamination [14]. DNA samples were stored at -70˚C until real-time PCR analysis.

TaqMan real-time PCR assay. Amplification and detection of *S. aureus* and methicillin-resistant staphylococci DNA by PCR were performed with the Smart Cycler thermal cycler (Cepheid, Sunnyvale, Calif.). The TaqMan real-time PCR assay has been designed as a multiplex analysis of *nuc* gene and *mecA* gene simultaneously using primers and probes (Table 3), and was synthesized by Eurogentec (Seraing, Belgium). The optimized PCR master mixture using the qPCR™Core Kit (Eurogentec) consisted of 300 nM of each primer, 200 nM of each probe, 4 mM $MgCl_2$, 0.4mM of each of the four dNTPs, 1.25 U of Hot GoldStar DNA polymerase, and 5 µl purified DNA in a total volume of 25 µl. The PCR cycling conditions included an initial denaturation step of 95ºC for 600 s followed by 40 cycles of 95ºC for 15 s and 60ºC for 1 min. The amplification mixture and a water blank used to prepare the reagents, and DNA extracted from methicillin-resistant *S. aureus* (MRSA) were included in every run as negative and positive PCR control, respectively.

The universal bacterial forward primer sequence was the reverse complement of the reverse primer described by Nadkarni et al [15] with some modifications (Table 3). The reverse primer and probe sequences (Table 3) were as described by Yang S et al [10]. The universal bacterial PCR assay was performed in a total volume of 25 µl containing 5 µl DNA extract, 200 nM of each primer and 150 nM of the probe (Eurogentec), and TaqMan Universal PCR Master Mixture (Applied Biosystems, Foster City, CA), including the enzyme uracil-N-glycosylase (UNG) to prevent carryover contamination from previous amplifications. The amplification and detection of DNA were performed in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The PCR cycling conditions were 50ºC for 2 min and 95ºC for 10 min, followed by 40 cycles of 95ºC for 15 s and 60ºC for 1 min. To test the PCR reaction mixture itself for contamination of bacterial DNA, four negative controls consisting of amplification mixture and sterile water instead of DNA template was included in each PCR.

Duplicate samples were routinely used for the real-time PCR assays and the presence of amplified products was confirmed when the fluorescent signal exceeded an automatic noise-based defined threshold (Ct).

Using QIAamp as DNA extraction method, the definition of positive, inconclusive, and negative results were as follows:

Positive: $Ct<[(Ct_{min}$ of negative DNA extraction controls) – 2SD]

Negative: $Ct\geq[(Ct_{mean}$ for negative DNA extraction controls) – 2SD]

Inconclusive: $[(Ct_{min}$ of negative DNA extraction controls) – 2SD]$<Ct<[(Ct_{mean}$ for negative DNA extraction controls) – 2SD]

Using MagNA Pure LC Microbiology Kit M^{GRADE} as DNA extraction method, the definition of positive, inconclusive, and negative results were as follows:

Positive: Ct<35

Negative: Ct>40

Inconclusive: 35<Ct<40

Results

Detection of S. aureus and methicillin-resistant staphylococci DNA by real-time PCR. DNA was extracted from 115 synovial fluids using the QIAamp® DNA Mini Kit and analysed for the *nuc* gene and *mecA* gene simultaneously. The protocol correctly detected all positive controls and no false positive signals were detected in the no-template controls. Four culture-positive *S.aureus* samples where all *nuc* PCR positive. None of the culture-negative specimens was PCR positive. One sample was *mecA* PCR positive. This bacterium was identified as *Staphylococcus epidermidis* by culture and reported as methicillin-resistant.

Detection of bacteria using QIAamp® extraction procedure and universal bacterial real-time PCR. Our PCR protocol was first assessed with genomic DNA extracts from 19 bacterial species commonly causing infections. All 19 bacterial species were detected by the universal bacterial real-time PCR (Table 2).
Extracted DNA from the 115 synovial fluids and controls were then run with universal primers and probe to detect the presence of any eubacterial DNA. This protocol detected all the positive controls, but also gave positive results for the no-template controls.
By this method bacteria were detected in all of the samples which were positive by culture and in seven of the culture-negative samples. This broad-range PCR protocol gave many results difficult to interpret, with many samples in a grey zone between positive and negative (referred to as inconclusive). Of these, 2 was culture positive, 36 was culture negative, and 1 was inconclusive (Table 4).

Detection of bacteria using M^{GRADE} extraction procedure and universal bacterial real-time PCR. The samples with enough synovial fluid left (67 samples) were finally used for DNA isolation with the MagNA Pure LC Microbiology Kit M^{GRADE}, and run with the universal bacterial PCR protocol. This protocol correctly detected all positive controls, and no controls were false positive.
The results from the two different DNA extraction procedures were finally compared with each other and the culturing results (Table 5).

Discussion

Contamination is a well known problem in bacterial detection using broad-range PCR. PCR is in theory capable of detecting one single copy of template DNA, making the demands for sterility difficult to maintain. In addition, the use of the highly preserved 16S rRNA-gene includes all bacteria. This creates a conflict between the desire to apply this enormous sensitive capacity, and the difficulties in detecting only the true pathogenous bacteria in a clinical sample.

Our samples were probably not contaminated during harvesting to a great extent. Samples were taken under ultra-sterile conditions in the operating theatre, after incision of the skin, but before opening of the joint capsule. Thus the operation had only lasted less than 5 minutes, and the chances of contamination of instruments and the operating field should be minimal compared to samples taken later in the revision (for instance from removed parts of the prosthesis itself which may take one hour or more).

We also experienced difficulties in eliminating bacterial DNA contamination in reagents used for PCR. To overcome this problem we tested several described successfull decontamination methods to destroy DNA when optimizing our PCR protocol. This included UV irradiation of the Taq-polymerase for various times, and prefiltration of the PCR mix prior to the addition of template-DNA using Amicon Microcon YM-100 centrifugal filter device (Millipore Co, Bedford, Mass). We also tested a “clean” Taq-polymerase (Ampli Taq LD, Applied Biosystems). It was not possible in this study to eliminate endogenous contaminating DNA from the PCR mixture with these methods without seriously compromising the efficiency of the PCR (data not shown).

Synovial fluid is an intricate, protein-rich material consisting of proteoglycans which makes the digestion of hydrolytic enzymes ineffective. It also contains inhibiting factors which can interfere with the PCR and it can be extremely viscose making the extraction and centrifugation difficult. When the synovial fluid is infected, the fluid becomes even more complex, making the process of using synovial fluid even more challenging [2].

With this knowledge of synovial fluid in mind we felt the need to control our DNA extraction using the QIAamp® DNA Mini Kit. The samples, some of them who were confirmed culture-positive with *S.aureus*, were therefore run with our standardized methicillin-resistant *S.aureus*-PCR to ensure true positive results.

Our results show that the *nuc* and *mecA* PCR successfully predicted the presence of respectively *S.aureus* and methicillin-resistant staphylococci in all samples. In this study the PCR results were concordant with the culture results and gave the results within 4 hours, compared with 2-3 days for the culturing. This corresponds with other studies on methicillin-resistant PCR-assays [16, 17]. It is important to be aware that in this PCR, detection of the *mecA* gen is not in conjunction with the S.aureus-specific genom fragment - the *nuc* gen. Therefore it is not possible to distinguish the presence of methicillin-resistent *S. aureus* (MRSA) from *mecA* positive strains of coagulase-negative staphylococcus spp (CNS) in samples containing both *S.aureus* and CNS.

We ended up using the TaqMan Universal PCR mix and molecular biology reagent water (Sigma-Aldrich Co) as negative PCR controls which gave successful results for these negative controls. However, after the extraction step with QIAamp® DNA Mini Kit, many negative

DNA extraction controls turned positive, rising suspicions of contamination with bacterial DNA in the QIAamp kit. This is concordant with other studies [18]. This further gave too many positive PCR results for the samples, making a cut-off of the Ct-values necessary.

Because of these problems with the QIAamp kit we tested the accessible samples again with the MagNA Pure LC Microbiology Kit M^{GRADE}. Using this DNA isolation kit no false positive negative extraction controls was observed, and so in this study, the MagNA Pure LC Microbiology Kit M^{GRADE} showed to be more reliable as extraction method.

Several studies operates with a detection threshold of the number of bacteria per cubic centimetre [7, 19]. The studies use different thresholds, making comparisons difficult. Clarke et al (2004) concludes that no conclusive data exist to indicate the level at which the detection threshold should be set. Our concern is that when choosing a threshold and thus reducing the sensitivity, one excludes samples with extreme low levels of bacteria. We do not yet know how low levels of bacteria are capable of causing the infections we are trying to find. In our study, we therefore do not operate with a detection threshold. This made the study vulnerable to laboratory-based false positive results.

The same problems arise when interpreting the universal PCR results. What Ct-values reflect correctly the shift between the low grade infections and the laboratory-based false positive results? More research is needed to make this gap between true low-grade infections and false background DNA results smaller. In this study our universal bacterial PCR detected bacteria in all of the samples which were positive by culture and in 58% (7 of 12) of the culture-negative samples (Table 4). The PCR-positive, culture-negative samples are unlikely to be positive due to contaminating bacterial DNA, as the criteria for positive samples were very stringent to make sure these samples were true positives. Our result verifies the suspicion that culturing does not detect all true pathogens.
Thirty-six samples found negative by culture gave PCR results within the “grey zone”. The total number of samples within the grey-zone by PCR was 41 of 115. Is this due to background DNA and contamination problems only, or is this group (also) containing true low-grade infections? With culture as the golden standard this question can still not be answered.

With our protocol none of the negative PCR results were culture-positive, indicating that the PCR protocol and cut off points used were adequate.
In this study synovial fluids with minimum three revision samples for culturing were included in the material. Zimmerli et al recommends that at least three intraoperative tissue specimens should be sampled for culturing [1]. We also defined culture positive samples as growth of the same bacteria in at least 2 different samples. This low number of samples in addition to the stringent positive criteria makes the interpretation of the results vulnerable. One can suspect a higher rate of false negative results with these criteria, and perhaps this contributes to the gap between the PCR and culture results.

Several other similar studies use other clinical examinations available to assist in predicting a diagnosis. In this study, we chose to only record the culture results, and not the other examinations available to make a diagnosis. Blood tests, isotope scanning, gram’s staining, histological analysis and culturing are all currently in use to diagnose the infections. The role and usefulness of each test have been reviewed, but no test, or set of tests, have showed required specificity and sensitivity to be considered reliable [1]. Histopathological findings vary with the different infections, depending on the type of bacteria, the duration of

the infection and whether the infection is polymicrobial [20]. The histopathological findings also give poor sensitivity, especially in cases of low-grade infections [21].

The clinical use of the PCR-techniques in near future will probably be with specific primers and probes when suspecting specific bacteria with high risk of complications, such as with the methicillin-resistant bacteria, where rapid and reliable detection is crucial. M*ecA*-PCR will provide a rapid answer for the bacterial sensitivity to penicillinase stable penicillins. Culturing can not be fully replaced with these methods. The detection of the resistance patterns will still be important to identify the bacterial sensitivity to different antimicrobial drugs. Perhaps will a combination of the two methods solve the problems with the detection of hip revision infections?
With PCR, antibiotic treatment will not affect the PCR-result because this technique is not dependent on in vitro growth of the organism. Thus one can start, or continue, antibiotic therapy while samples are taken and the assay is analysed. This is important especially when identifying highly pathogenic bacteria which can give life threatening infections.

This study gave no negative PCR results on the culture-positive samples. This gives the protocol a potential as a rapid detection of true “non-infected”. This can be a valuable result when the other tests, such as CRP and ESR are inconclusive. Because of the short analysing time one can thus assume that the protocol can be useful as part of a preoperative assessment.

Our goal in this study was to develop a better and more reliable diagnostic method for the complicated hip replacement infections. The contamination issues prove this goal yet difficult to achieve and more research is needed to overcome this obstacle. Until the problems with contamination are solved, perhaps the synovial fluid should be examined with both PCR and culturing.

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Table 1. Bacteriology of prosthetic joint infection, pathogens and frequency (%) [a]

Coagulase-negative staphylococci	22%
Staphylococcus aureus	22%
Viridans streptococci	9%
Beta-hemolytic streptococci gr A, B, G	5%
Enterococci	7%
Gram-negative aerobic bacilli	25%
Anaerobes	10%

[a]Mandell, G.L., et al., *Mandell, Douglas, and Bennett's principles and practice of infectious diseases - 5th ed.* 2000.

Table 2. Bacterial species detected by the TaqMan real-time PCR using the universal probe and primer set

Bacterial species	Isolate type or ATCC no.
Streptococcus pyogenes	19615
β-hem streptococci	Clinical isolate
Staphylococcus epidermidis	12228
Enterococcus faecalis	51299
Streptococcus pneumoniae	49619
Haemophilus influenzae	Clinical isolate
Neisseria meningitidis	Clinical isolate
Staphylococcus aureus	Clinical isolate
Streptococcus sanguis	Clinical isolate
Propionibacterium acnes	Clinical isolate
Bacillus subtilis	6633
Klebsiella pneumoniae	700603
Enterobacter cloacae	Clinical isolate
Serratia marcescens	Clinical isolate
Pseudomonas aeruginosa	Clinical isolate
Streptococcus oralis	Clinical isolate
Escherechia coli	12900
Klebsiella oxytoca	Clinical isolate
Staphylococcus epidermidis	Clinical isolate

Table 3. Oligonucleotide sequences of primers and probes used in this study

Oligonucleotide	Target gene	Oligonucleotide sequence (5´→3´)	Fluorophores
S. aureus	*nuc*		
Forward primer:		ATGGACGTGGCTTAGCGTAT	
Reverse primer:		TTTTTCGCTTGTGCTTCACTT	
Probe:		AGCAACTTTAGCCAAGCCTTGACGAAC	Yakima, Darquencher
Methicillin-resistance	mecA		
Forward primer:		TCCAGGAATGCAGAAAGACC	
Reverse primer:		ACATTCTTTGGAACGATGCC	
Probe:		TGGCCAATTCCACATTGTTTCGG	FAM, Darquencher
Bacteria	16S rDNA		
Forward primer:		AGGATTAGATACCCTGGTAGTCCA	
Reverse primer:		TGCGGGACTTAACCCAACA	
Probe:		CACGAGCTGACGACARCCATGCA	FAM, Darquencher

Table 4 Comparison of universal bacterial real-time PCR[a] with culturing

PCR / Culturing	Positive	Inconclusive	Negative	Total
Positive	5	2	0	**7**
Inconclusive	0	1	1	**2**
Negative	7	36	63	**106**
Total	**12**	**39**	**64**	**115**

[a] using QIAamp® as extraction method

Table 5 Evaluation of two different DNA-extraction methods[a], using universal bacterial real-time PCR and compared with culture results[b]

M^{GRADE} / **QIAamp®**	**PCR positive**	**PCR inconclusive**	**PCR negative**	**Total**
PCR positive	5 (3,0,2)	4 (0,0,4)	3 (2,0,1)	**12** **(5,0,7)**
PCR inconclusive	4 (0,0,4)	21 (2,0,19)	12 (0,1,11)	**37** **(2,1,34)**
PCR negative	0	12 (0,1,11)	6 (0,0,6)	**18** **(0,1,17)**
Total	**9** **(3,0,6)**	**37** **(2,1,34)**	**21** **(2,1,18)**	**67** **(7,2,58)**

[a] MagNA Pure LC Microbiology Kit M^{GRADE} and QIAamp® DNA Mini Kit (Qiagen).
[b] Culture results referred in parentheses with positive, inconclusive and negative results respectively